Experimental Parasitology 128 (2011) 272–278

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Trypanosoma brucei s.l.: Microsatellite markers revealed high level of multiple genotypes in the mid-guts of wild tsetse flies of the Fontem sleeping sickness focus of Cameroon

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ARTICLE INFO

Article history:
Received 4 June 2010
Received in revised form 25 October 2010
Accepted 21 February 2011
Available online 2 March 2011

Keywords:
Microsatellite markers
Sleeping sickness
Trypanosoma brucei
Tsetse fly mid-gut
Multiple genotypes

ABSTRACT

To identify *Trypanosoma brucei* genotypes which are potentially transmitted in a sleeping sickness focus, microsatellite markers were used to characterize *T. brucei* found in the mid-guts of wild tsetse flies of the Fontem sleeping sickness focus in Cameroon. For this study, two entomological surveys were performed during which 2685 tsetse flies were collected and 1596 (59.2%) were dissected. Microscopic examination revealed 1.19% (19/1596) mid-gut infections with trypanosomes; the PCR method identified 4.7% (75/1596) infections with *T. brucei* in the mid-guts. Of these 75 trypanosomes identified in the mid-guts, *Trypanosoma brucei gambiense* represented 0.81% (13/1596) of them, confirming the circulation of human infective parasite in the Fontem focus. Genetic characterization of the 75 *T. brucei* samples using five microsatellite markers revealed not only multiple *T. brucei* genotypes (47%), but also single genotypes (53%) in the mid-guts of the wild tsetse flies. These results show that there is a wide range of trypanosome genotypes circulating in the mid-guts of wild tsetse flies from the Fontem sleeping sickness focus. They open new avenues to undertake investigations on the maturation of multiple infections observed in the tsetse fly mid-guts. Such investigations may allow to understand how the multiple infections evolve from the tsetse flies mid-guts to the salivary glands and also to understand the consequence of these evolutions on the dynamic (which genotype is transmitted to mammals) of trypanosomes transmission.

1. Introduction

Human African Trypanosomiasis (HAT) is an important public health disease in sub-Saharan Africa, and is responsible for a considerable degree of suffering and mortality in countries where it is endemic. Some 60 million people in 36 countries are at risk, with the number of new cases reported per year to 10,000 and the new estimated cumulative infection cases of about 50,000–70,000 (Simarro et al., 2008). HAT is caused by the protozoan parasite *Trypanosoma brucei* and is transmitted by the tsetse flies (genus Glossina). *Trypanosoma brucei* s.l. are hemoflagellated parasites that infect humans as well as a variety of domestic and wild mammalian species in sub-Saharan Africa. Three subspecies of *T. brucei* are currently recognized (Hoare, 1972): *T. b. brucei*, which is defined as infecting animals but not humans and is present throughout the tsetse distribution area in Africa; *Trypanosoma brucei gambiense*, which is infective for humans in West and Central Africa and whose infection results in chronic human sleeping sickness; and *T. b. rhodesiense*, which is defined as human infective and localized in East and south Africa and causes acute sleeping sickness.

One of the characteristic features of the history of HAT is the association of specific areas or foci with the disease and a cycling between periodic devastating epidemics interspersed with long periods of low-level endemicity. The key for the control of HAT depends on the understanding of mechanisms responsible for the epidemic or endemic evolutions of the disease. To determine the nature of these mechanisms, it is necessary to understand how a focus of HAT originates, which factors favor the stability of these foci over long periods, how a HAT epidemic develops from low endemic levels within a focus and which factors are responsible for disease outbreaks. For a couple of decades, much efforts have been focussed on the genetic characterization of *T. brucei* s.l. in order to identify animal reservoirs of human infective parasites or to identify parasite genotypes that may be responsible for endemic or epidemic evolution of the disease. To undertake these characterizations, several biochemical and molecular biology techniques including isoen-

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doi:10.1016/j.exppara.2011.02.023

zyme electrophoresis (Godfrey and Kilgour, 1976; Godfrey et al., 1987), Restriction Fragment Length Polymorphism analysis (RFLP) (Kanmogne et al., 1996a), Random Amplification of Polymorphic DNA (RAPD) (Stevens and Tibayrenc, 1995; Kanmogne et al., 1996b), minisatellites and microsatellites DNA amplification techniques (MacLeod et al., 2000; Biteau et al., 2000; Koffi et al., 2007, 2009; Simo et al., 2010), Mobile Genetic Element PCR (MGE-PCR) (Tilley et al., 2003; Simo et al., 2005), and Amplified Fragment Length Polymorphism (AFLP) (Agbo et al., 2002; Simo et al., 2008) have been used. These techniques have generated important epidemiological information on the relationships between trypanosome strains and their potential role in the heterogeneity of the disease in different foci, and also in the generation and maintenance of HAT foci (Tilley et al., 2003; Njiokou et al., 2004; Simo et al., 2005, 2008). Furthermore, these techniques demonstrated the existence of gene exchanges between *T. brucei* strains as well as the presence of multiple genotype infections of *T. brucei* s.l. in vertebrate hosts (Tait, 1980, 1983; Truc et al., 2002; Jamonneau et al., 2003) as well as in tsetse flies (MacLeod et al., 1999; Balmer and Tostado, 2006). Despite these interesting results, it remains clear that a better understanding of the transmission and the spread of HAT as well as the dynamics of its endemic or epidemic evolution requires that the different strains infecting a host can be distinguished. Since multiple strain infections of *T. brucei* have been reported in vertebrate hosts and tsetse flies, they may have important epidemiological significance due to the fact that they are able to generate new genotypes from genetic exchange that occurs in tsetse flies (Gibson and Bailey, 1994; Gibson et al., 1997; Jenni et al., 1986). Moreover, mixed infections have become an issue of growing importance because of its recognized role in disease evolution (Ebert and Mangin, 1997; Read and Taylor, 2001), drug resistance (de Roode et al., 2004), and transmission dynamics (Gupta et al., 1994; Hudson et al., 2002; Plebanski et al., 1999). In HAT, considerable efforts have been focussed on the characterization of *T. brucei* isolates from vertebrate hosts as well as from tsetse flies. Previous studies on the genetic characterization of trypanosomes were performed on parasites isolated from tsetse flies or mammals. From such isolates, unique individuals are generally isolated and their complete genotypes can easily be obtained. However, during the isolation process, a sub sampling of existing genetic diversity occurs inside the host (Koffi et al., 2009), resulting in a possible information loss. The need to undertake investigations on field samples without isolation was a challenge until a couple of years ago. For such investigations, highly polymorphic, relatively easy to score, non-coding sequences, specific and reliable tools like microsatellite markers were developed. During the last decade, several polymorphic microsatellite markers specific for *T. brucei* complex species have been developed for the characterization of trypanosome isolates and for the assessment of the population structure and reproductive mode of *T. b. gambiense* group 1 (Biteau et al., 2000; Koffi et al., 2007, 2009; Morrison et al., 2008) and also for the construction of genetic maps of these parasites (MacLeod et al., 2005; Cooper et al., 2008).

In this study, microsatellite markers were used to characterize *T. brucei* s.l. circulating in the mid-guts of wild tsetse flies from the Fontem sleeping sickness focus in order to identify *T. brucei* genotypes which are potentially transmitted in this HAT focus.

2. Materials and methods

2.1. Study zone

This study was performed in five villages (Besali, Bechati, Folepi, Agong and Menji) of the Fontem sleeping sickness focus (5°40′12N, 9°55′33E) (Fig. 1). Situated in the South-West Region of Cameroon, this focus is characterized by a tropical humid climate with varied topography made up of hills and valleys through which several high speed rivers flow (Asonganyi et al., 1990). The main population activities are subsistence agriculture, palm oil extraction, animal husbandry and small scale poultry farming. The dense population of humans, domestic animals and tsetse flies are found scattered in the pre-forest/forest vegetation of the valleys and hills (Asonganyi et al., 1990). *Glossina palpalis palpalis* is the main vector of sleeping sickness in this focus (Morlais et al., 1998). No vector control activities have been implemented in this area for more than 15 years. Studies on animal reservoir revealed *T. b. gambiense* in pigs (Nkinin et al., 2002; Simo et al., 2006). Previously, the Fontem focus was among the most active sleeping sickness foci in Cameroon (Asonganyi and Ade, 1992). From 1997 to 2000, four medical surveys carried out in this focus revealed a prevalence of 0.05% in 16,000 inhabitants examined (OCEAC, unpublished data).

2.2. Entomological surveys

During this study, two entomological surveys were carried out, in November (dry season) and April (rainy season), in 5 villages of the Fontem sleeping sickness focus (Fig. 1). In each village, pyramidal traps (Gouteux and Lancien, 1986) were set up in various tsetse fly favorable biotopes. Each trap was numbered and geo-referenced using the Global Positioning System. Each trap remained for four consecutive days and during this period, tsetse flies were collected twice (between 8 and 10 AM and between 2 and 4 PM) each day. For each fly caught, the trap number, date, village and the fly number were recorded. All flies caught underwent sex and species identification. Living and teneral flies (young fly which has never taken a blood meal) were also identified. All living flies were dissected in a drop of 0.9% saline solution using a stereo microscope and their mid-guts were examined under a light microscope (magnification × 100) for the presence of trypanosomes. After the examination, the mid-guts were collected separately into microtubes containing ethanol (95°). These microtubes were kept at room temperature during the field surveys, and stored in the laboratory at −20 °C until use.

2.3. DNA extraction

DNA was extracted using Chelex 100 (Walsh et al., 1991). Briefly, 1 ml of 5% Chelex was put into micro tubes containing tsetse fly mid-gut and the mixture was incubated in a water bath for 1 h at 56 °C, then for 30 min at 100 °C. The tubes and their contents were centrifuged for 10 min at 14,000 rpm. The supernatant was carefully collected and used as DNA template for the PCR reactions.

2.4. Identification of T. brucei s.l. and T. b. gambiense group 1

For each sample (tsetse mid-gut), *T. brucei* s.l. specific primers (TBR1: 5′-CGAATGAATATTAAACAATGCGCAG-3′ and TBR2: 5′-AGAACCATTTATTAGCTTTGTTGC-3′) were used to investigate the presence of this trypanosome species (Masiga et al., 1992). A second PCR was done on all *T. brucei* s.l. positive samples in order to identify *T. b. gambiense* infections. This second PCR was performed using primers (TRBPA1: 5′-GCGCCGACGATACCAATGG-3′ and TRBPA2: 5′-AACGGATTTCAGCGTTGCAG-3′) characteristic of group 1 *T. b. gambiense* (Herder et al., 2002). For the identification of *T. brucei* s.l. as well as *T. b. gambiense* infections, the amplification reactions were performed as described by Herder et al. (2002) using 5 μl of DNA extract.